ELECTROPHORETIC PURIFICATION OF RECOMBINANT GREEN FLUORESCENT PROTEIN FROM INTACT *Escherichia coli* CELLS IN CONTINUOUS BUFFER SYSTEM

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ABSTRACT

Green fluorescent protein (GFP) is a protein that consists of 27 kDa protein of 238 amino acid residues. GFP emits bright green fluorescence light when exposed to blue or ultraviolet light. GFP has been used as a marker for the gene expression visualization, protein localization in living and fixed tissues as well as for protein targeting in intact cells and organisms. A direct purification method was developed to purify the recombinant GFP from intact *Escherichia coli* (*E. coli*) cells using preparative native polyacrylamide gel electrophoresis (n-PAGE) in continuous buffer system. 100 µL of 12% (w/v) polyacrylamide gel was used to study the effect of biomass concentration and the effect of resolving gel height on the preparative n-PAGE. The amount of purified GFP was determined by using the gel-based imaging method and the Lowry protein determination method to determine the purity and yield of the recovered GFP. The optimal biomass concentration in the feedstock was found at 15% (w/v) with 62.5% of purity. The purity of GFP slightly reduced when the biomass concentration increased to 25% (w/v). Meanwhile, 89% of purity was achieved when 1 cm of resolving gel was employed in preparative n-PAGE. The purity of the GFP decreased when the gel height increased to 2.5cm. However, the percentage of the yield in this study was unable to determine since the calculation was completely offset.

ABSTRAK

Protein pendarflour hijau (GFP) adalah protein yang mengandungi 27 kDa dengan baki 280 asid amino. GFP mengeluarkan warna hijau terang apabila terkena sinaran cahaya biru atau cahaya UV. GFP telah digunakan sebagai penanda genetik dalam pemerhatian visual genetik, penentuan protein dalam tisu-tisu hidup termasuk sel dalam sesuatu organisma tersebut. Satu cara penulenan GFP secara langsung telah dibangunkan untuk menulenkan GFP rekombinan yang berasal daripada sel *Escherichia coli* (*E. coli)* dengan menggunakan Elektroforesis sediaan dengan gel poliakrilamida asli (n-PAGE) dalam sistem buffer yang berterusan. Sebanyak 100 µL 12% (w/v) gel poliakrilamida telah digunakan untuk mengkaji kesan kepekatan dan ketinggian gel poliakrilamida terhadap n-PAGE. Bilangan GFP yang tulen dianalisa dengan menggunakan analisis pengimejan berasaskan gel dan cara penentuan bilangan protein Lowry untuk menentukan ketulenan dan hasil GFP. Ketulenan optima bagi GFP untuk kesan kepekatan dalam 15% (w/v) biojisim ialah 62.15%. Ketulenan GFP menurun apabila kepekatan dalam suapan meningkat kepada 25% (w/v). Manakala kesan ketinggian gel polyacrylamide, sebanyak 89% ketulenan GFP telah diperolehi apabila ketinggian gel poliakrilamida sebanyak 1 cm. Ketulenan GFP menurun apabila ketinggian gel poliakrilamida meningkat kepada 2.5 cm. Walaubagaimanapun, bilangan hasil GFP tidak dapat ditentukan dalam kajian ini memandangkan bacaan untuk hasil GFP adalah tidak tepat.

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LIST OF ABBREVIATIONS

kDa	kilo Daltons
µL	microliters
µg	micrograms
w/v	weight over volume
V	volt
cm	centimetres
A	amperes
°C	celcius
g/L	grams/litre
µg/mL	micrograms per millilitre
mL	millilitre
rpm	revolutions per minute
mM	millimolar
M	molarity
OD_{600}	optical density at 600 nm
nm	nanometres
mA	milliamperes
W	watt
mm	millimetres
X	times
hrs	hours

LIST OF ABBREVIATIONS

DNA	deoxyribonucleic acid
HPLC	high-performance liquid chromatography
n-PAGE	native polyacrylamide gel electrophoresis
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
TPP	three phase partitioning
IEF	isoelectric focusing
IPTG	isopropyl β-D-1-thiogalactopyranoside
Tris	2-Amino-2-hydroxymethyl-propane-1,3-diol
TEMED	N,N,N’,N’-tetramethylethylenediamine

1 INTRODUCTION

1.1 Motivation and statement of problem

For over decades, gene mapping was limited in most organisms by traditional genetic markers which include genes that encode easily observable characteristics such as blood types or seed shapes. Genetic marker is a gene or DNA sequence with a known location on a chromosome that can be used to identify individuals or species ("LoveToKnow", 2013). Genetic marker are widely apply in medical field where genetic marker was used to study disease and improve human health through the use of technologies that integrate the entire genome (Gibbons *et al.*, 2004). Besides that, Neale *et al.* (1992) used the genetic marker in their forest tree improvement research. Green fluorescent protein (GFP) is one of the genetic marker example that used as a reporter in cell and molecular biology. GFP was discovered by Osamu Shimomura in the 1960s where the gene was first isolated from the jellyfish, *Aequorea victoria*. Unlike most of the genetic marker, GFP can be fused with other proteins without altering other proteins function. GFP emits bright green fluorescence when it exposes under blue or ultraviolet light (Tsien, 1998). It has 27 kDa proteins of 238 amino acid residues. Asides from being genetic marker, GFP is also used as a genetic fusion partner. Green fluorescent chimera was created to host proteins in order to monitor their localization (Tsien, 1998). Kac (2000) had successful fused GFP with a rabbit for art purposes and social commentary.

Purification process had been crucial for researches to have detailed studies on the function of targeting protein (Young, 2006). Yield and purity of a protein usually depends on purification method. For instance, intracellular protein purification usually requires preliminary cell disruption to release the intracellular protein from intact cells before undergoes subsequent purification process. Cell disruption may cause the protein degradation, thus high losses of the products (Ho *et al.*, 2008).

Due to this problem, a direct purification method had been developed by Chew *et al.* (2009) for purification of recombinant GFP from intact *Escherichia coli* cells. A homemade apparatus of preparative native polyacrylamide gel electrophoresis (n-PAGE) is used to combine the cell disruption, clarification, concentration, and separation steps into a single purification step. In their study, a discontinuous buffer system was employed which consists of 2 layers of gel in the n-PAGE. Continuous buffer systems use the same type of buffer, at constant pH, sample,

and electrode reservoirs (Garfin, 2003). Compare to discontinuous buffer system, continuous buffer system can use almost any type of buffer (Garfin, 2003) and this can simplify the process of preparative n-PAGE. Therefore, this study aims to develop purification method for purifying the recombinant GFP from intact *E. coli* BL21 (DE3) cells using preparative n-PAGE in a continuous buffer system.

1.2 Objectives

The following are the objectives of this research:

i) To develop purification method for purifying the recombinant GFP from intact *E. coli* BL21 (DE3) cells using preparative n-PAGE in a continuous buffer system.
ii) To study the effect of biomass concentration on the preparative n-PAGE.
iii) To study the effect of height of resolving gel on the preparative n-PAGE.

1.3 Scope of this research

The following are the scope of this research:

i) 100 µL of 12% (w/v) polyacrylamide gel was used to study the effect of biomass concentration and the effect of resolving gel height on the preparative n-PAGE.
ii) The preparative n-PAGE was runs at constant voltage of 140V.
iii) The purified GFP was analysed by using gel-imaging method.
iv) The amount of total GFP was determined by using Lowry protein assay.

1.4 Main contribution of this work

The following are the contributions:

i) An integrated purification process was developed where it combines the cell disruption, clarification, concentration, and separation steps into a single purification step.
ii) A continuous buffer system was employed where it was simple and cheaper compared with existence preparative n-PAGE.

1.5 Organisation of this thesis

The structure of the reminder of the thesis is outlined as follow:

Chapter 2 covers the information about the characteristic and the application of the green fluorescent protein. Besides, the descriptions about the available purification methods for green fluorescent protein are reviewed. The type of polyacrylamide gel electrophoresis is described in the end of this chapter.

Chapter 3 describes the methodology and chemicals used in this study. *E. coli* strain BL21 (DE3) carrying the pRSETGFP plasmid encoding the GFP is used to produce the GFP. Then, 100 µL of feedstock containing *E. coli* biomass was loaded into 12% (w/v) polyacrylamide gel column and runs by using homemade apparatus of preparative n-PAGE at room temperature under constant voltage of 140V. The amount of purified GFP was analysed and determined using gel based imaging method while the total amount of purified GFP was determined by Lowry protein assay.

Chapter 4 provides standard calibration curve for Lowry protein assay and gel imaging analysis. 100 µL of feedstock containing E. coli biomass was loaded into 1.5 cm of gel column height in order to study the effect of biomass concentration on purification of preparative n-PAGE. Meanwhile, 100 µL of feedstock containing 20%(w/v) of *E. coli* biomass was loaded into 12% (w/v) polyacrylamide gel in order to study the effect of polyacrylamide gel height on purification of preparative n-PAGE. Both parameter runs at room temperature under constant voltage of 140V. The effect of the biomass concentration and the height of polyacrylamide gel on purification of preparative n-PAGE were studied and discussed

Chapter 5 draws together a conclusion of the thesis and outlines the future work which may improvise the purification of GFP in continuous buffer system.

2 LITERATURE REVIEW

2.1 Overview

This chapter covers the information about the characteristic and the application of the green fluorescent protein. Besides, the descriptions about the available purification methods for green fluorescent protein are reviewed. The type of polyacrylamide gel electrophoresis is described in the end of this chapter.

2.2 Green Fluorescent Protein (GFP)

Since the discovery of GFP by Osamu Shimomura in the 1960s, the research of GFP has been begun from cloning to purification of the protein. GFP was first isolated from a bioluminescent jellyfish, *Aequorea victoria* (Figure 2.1). However, the green-light of the GFP only activate when the GFP absorbed the blue-light produced by aequorin upon the calcium binding (Chalfie *et al.* 1994). GFP was successfully cloned and expressed the protein in *Escherichia coli* (Figure 2.2) and *Caenorhabditis elegans* in Martin Chalfie's lab (Tsien, 1998). These bacteria expressed the green fluorescent when it was induced with isopropyl-β-thiogalactoside (IPTG) (Chalfie *et al.*, 1994). GFP is a stable, water-soluble, and globular protein of molecular weight 27 kDa with isoelectric point near pH 5.3 (Ward, n.d). It is comprised of 238 amino acids (Yang, Moss, and George, 1996). The structure of the GFP was named beta-can by Yang *et al.*, 1996. From Figure 2.3, 11 antiparallel beta strands (green) form a very compact cylinder on the outside of the GFP structure. While inside the beta-structure, there is an alpha-helix (light blue) and in the middle of which is the chromophore (yellow). The middle structure responsible for the GFP to release its green fluorescence (Yang *et al.*, 1996).

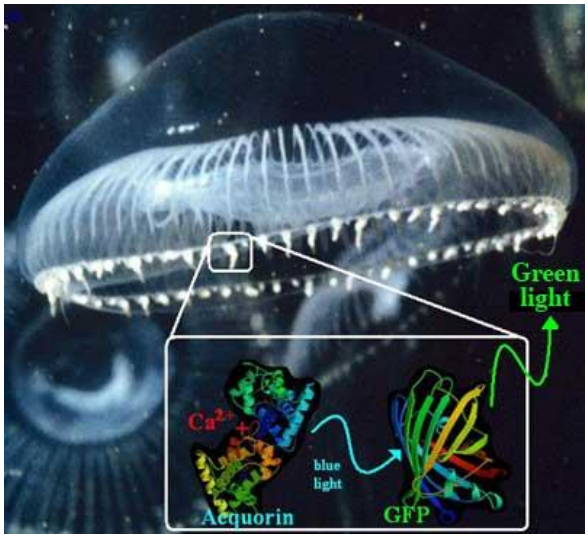

Figure 2.1: *Aequorea Victoria*

Source: Zimmer (2013)

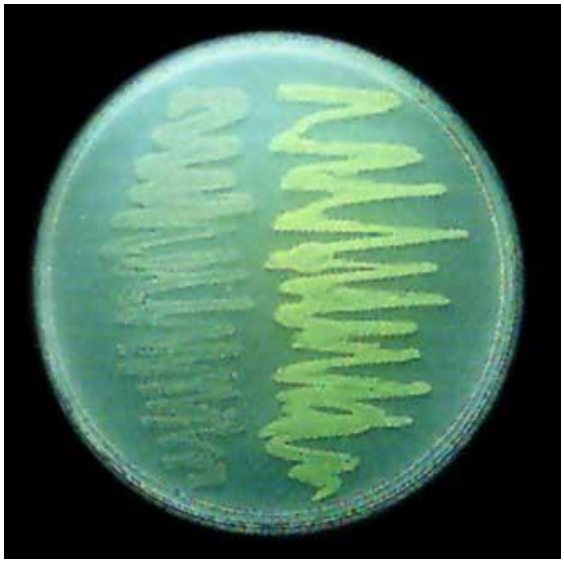

Figure 2.2: Expression of GFP in E. coli. The bacteria on the right side of the figure have the GFP expression plasmid. Cells were photographed during irradiation with a hand-held long-wave UV source.

Source: Chalfie *et al.* (1994)

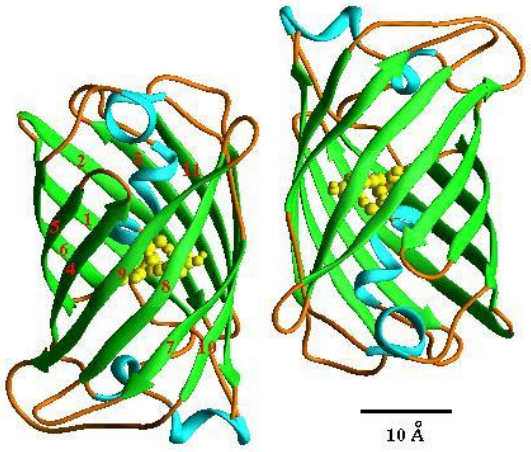

Figure 2.3: The structure of GFP: beta-can

Source: Yang *et al.* (1996)

2.3 Application of Green Fluorescence Protein

The fusion of the GFP to a protein rarely affect the proteins activity or mobility and it is nontoxic (Zimmer, 2002). Furthermore, GFP is resistant to heat, alkaline pH, detergents, photobleaching, chaotropic salts, organic salts, and many proteases (Ehrmann, Scheyhing, and Vogel, 2001). This make the GFP become favourable protein in many application.

2.3.1 GFP as Reporter Gene

GFP as a reporter gene was the first application to detect gene expression in vivo (Chalfie *et al.*, 1994). The GFP used to monitor gene expression under the control of a promoter of interest to measure the GFP fluorescence which directly indicates the gene expression in the cells (Zimmer, 2002). For example, GFP was particularly successful at confirming the pattern of expression of the mec-7 promoter, which drives the formation of β-tubulin in a limited number of mechanosensory neurons (Tsien, 1998). However, the GFP required strong promoter to drive sufficient expression for detection since there is no signal amplication (Tsien, 1998). This is due to each molecule of GFP has only one chromophore which lower its sensitivity (Zimmer, 2002). In order to overcome the problem, Tsien (1998) suggested using reporter gene products that can enzymatically catalyse a large change in the fluorescence of substrates that can be loaded into intact, fully viable cells. Another alternative suggested by Zimmer (2002) was to use high sensitivity photon counting devices.

2.3.2 Fusion Tags

A chimera was the resultant of a fusion of cloned gene and GFP using standard subcloning techniques (Zimmer, 2002). GFP fusion tags were used to visualise dynamic cellular events and to monitor the protein localization (Tsien, 1998). The fusion protein can maintain its normal functions and protein localization since the chromophore in GFP was produced in vivo (Zimmer, 2002). Due to this advantage, many major oraganelles were successfully fused and the migration of GFP from cell to cell had been observed (Tsien, 1998). The fusion between GFP and the protein of interest can be attempted at either the amino or carboxyl terminus of the host protein (Zimmer, 2002). There were 10 possible topologies of GFP and their chimeras with other proteins in Zimmer (2002) report.

2.3.3 Other GFP applications

The rigid shell in GFP surrounding the chromophore enables it to be fluorescent and protects it from photobleaching but also hinders environmental sensitivity. Due to this features, GFPs that act as indicators of their environment have been created by combinations of random and directed mutagenesis. Several applications based on GFP indicator for calcium, pH, metal and protease has been reported in Zimmer (2002) and Tsien (1998). These indicators are used based on fundamental technique called Fluorescence Resonance Energy Transfer (FRET). FRET is a nonradiative exchange of energy from an excited donor fluorophore to an acceptor fluorophore that is within 100 A from the donor (Zimmer, 2002). FRET is used to study the protein-protein interaction, determination of calcium concentration (Zimmer, 2002), and metal release monitoring (Tsien, 1998). Blue, green, cyan and yellow fluorescent proteins are the best FRET pairs because of their emission and excitation spectra (Zimmer, 2002).

In the recent study, a biological cell laser based on GFP has been invented by Gather and Yun (2011) to overcome the limited penetration of light in biological tissue. Compare to previous laser materials, GFP are biologically producible, biocompatible and bioabsorbable which made GFP solutions suited to generating stimulated emission and laser light from and within living organisms (Gather and Yun, 2011).

2.4 Available Purification Methods

Many recombinant GFP methods had been purposed such as organic extraction (Yakhninet *et al.*, 1998), three phase partitioning (Jain, Singh and Gupta, 2004), immobilized metal affinity chromatography (Noubhani *et al.*, 2002), anion exchange chromatography (Cabanne *et al.*, 2005), monoclonal antibody affinity chromatography (Zhuang *et al.*, 2008), hydrophobic interaction chromatography (McRae, Brown, and Bushell, 2005), chromatofocusing with a pH gradient (Narahari *et al.*, 2001), size exclusion chromatography and ion exchange HPLC (Deschamps, Miller, and Ward, 1995), and aqueous extraction followed by metal ions precipitation (Jain, Teotia, and Gupta, 2004).

2.4.1 Three phase partitioning (TPP)

Dennison and Lovrien (1997) described the three-phase partitioning (TPP) as a batch method with three stages that usually for rapid purification of proteins. This method required high concentration of well-buffered aqueous ammonium sulphate together with an equal volume of water-miscible aliphatic alcohol (Ward, n.d). According to Gupta and Sharma (2001) in the pectinase purification, the TPP method only involves 2 major steps, including the addition of ammonium sulphate to desired level and centrifugation in order to facilitate separation process. The GFP forms dimers when at high concentrations of ammonium sulphate, thus the GFP stabilized by hydrophobic and intermolecular interactions (Ward, n.d).

2.4.2 Monoclonal antibody coupled affinity

Monoclonal antibodies are made by identical immune cells where all the clones are from a unique parent cell, in contrast to polyclonal antibodies which are made from several different immune cells (Schwaber and Cohen, 1973). It binds to the same epitope because it has monovalent affinity (Schwaber and Cohen, 1973). Therefore, the monoclonal antibodies are able to detect and purify a substance. Zhuang *et al.* (2008) used female BALB/mice to produce monoclonal antibodies where the mice were immunized for 2 weeks. After the immunoprecipitation process, the GFP fusion protein was purified under an affinity column chromatography. This method has successfully purified the GFP with a purity of 97% and yield of 90% (Zhuang *et al.*, 2008).

2.5 Gel electrophoresis

Gel electrophoresis is a technique whereby charged molecules are separated by the used of an electric field (Garfin, 2003). The charged molecules tend to migrate towards an opposite charge during electrophoresis. This process usually carried out in an aqueous solution. Polyacrylamide is the matrix that commonly used in protein gel electrophoresis. The mobility of a protein depends on its charge, size, and shape. However, the mobility of the protein can be influence by pH change and types of counter ions and denaturants (Garfin, 2003). Researchers usually use gel electrophoresis for protein analysis and purification purpose. Gel electrophoresis can be categorized into 2 types, one dimensional and two dimensional.

2.5.1 One dimensional polyacrylamide gel electrophoresis (1D-PAGE)

2.5.1.1 Native-Polyacrylamide Gel Electrophoresis (n-PAGE)

The protein in native state are properly folded and electrophorese without being denature by denaturant ("Alliance Protein Laboratories Inc.," 2012). N-PAGE is used to separate proteins in their native states according to difference of charge density. N-PAGE can runs either in continuous buffer system or in discontinuous buffer system. As usual, the mobility in n-PAGE also depends on both of the protein's charge. However, the charges also depend on the amino acid composition of the protein ("Thermo Fisher Scientific Inc.," 2012). Proteins with compact conformations have higher mobility while the larger structures have lower mobility ("Thermo Fisher Scientific Inc.," 2012). This PAGE is suitable to use in preparation of purified and active proteins since this PAGE did not denature protein. The external electric field causes the cells to release its intracellular contents (Chew *et al.*, 2009). Then, the preparative n-PAGE purification takes place as the GFP migrate to the bottom end of the polyacrylamide gel.

2.5.1.2 Isoelectric Focusing (IEF)

IEF is employed when the conditions is desirable to maintain biological activity or antigenicity without denature the protein (Garfin, 2003). In IEF, proteins are separated by electrophoresis in a pH gradient based on their isoelectric point, pI (Garfin, 2003). The protein will move towards the more negative end of the gel if the proteins are positively charged and vice versa when the proteins are positively charged (Garfin, 2003). The protein molecule will accumulate at its isoelectric point and form a sharp band when the protein molecules carry no net charge.

2.5.1.3 Sodium Dodecyl Sulfate- Polyacrylamide Gel Electrophoresis (SDS- PAGE)

SDS-PAGE is a very common method for electrophoresis for separating proteins. Like n-PAGE, SDS-PAGE also consists of two different sub-gels, a stacking and a resolving gel. As the proteins enter the resolving gel, the polyacrylamide slows the larger molecules from migrating as fast as smaller molecules so creating separation based on mass (Raymond and Wientraub, 1959). However, SDS-PAGE requires the protein to denature to their constituent's polypeptide chains (Figure 2.4). So, it was suitable to determine the purity in purification process and to estimate the molecular weights of proteins (Garfin, 2003).

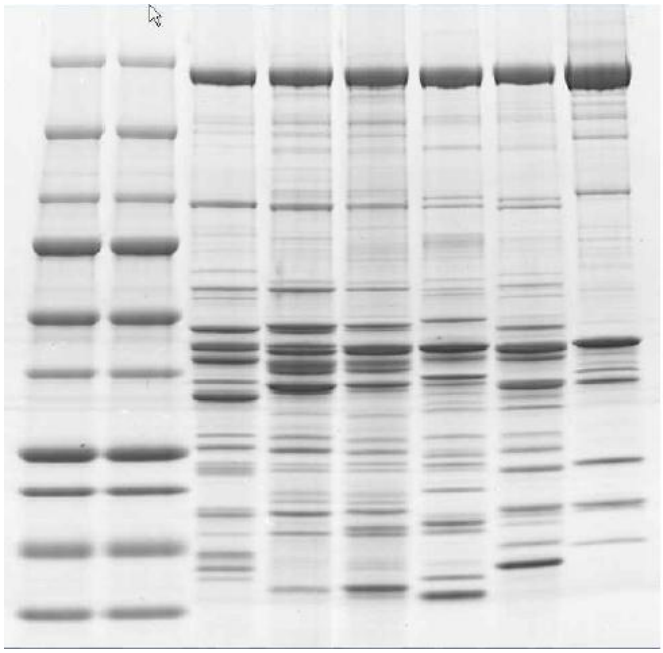

Figure 2.4: A typical analytical SDS PAGE

Source: Garfin (2003)

2.5.2 Two dimensional polyacrylamide gel electrophoresis (2D-PAGE)

2D-PAGE is a technique that combines IEF with SDS-PAGE (Garfin, 2003). It is a very efficient separation and sensitive detection for a protein (Issaq and Veenstra, 2008). Proteins were resolved on a gel using isoelectric focusing, which separates proteins in the first dimension according to their isoelectric point, followed by electrophoresis in a second dimension in the presence of sodium dodecyl sulfate, which separates proteins according to their molecular mass (O'Farrell, 1975).

3 MATERIALS AND METHODS

3.1 Overview

In this chapter, the overview of the methodology was summarized as following process flow:

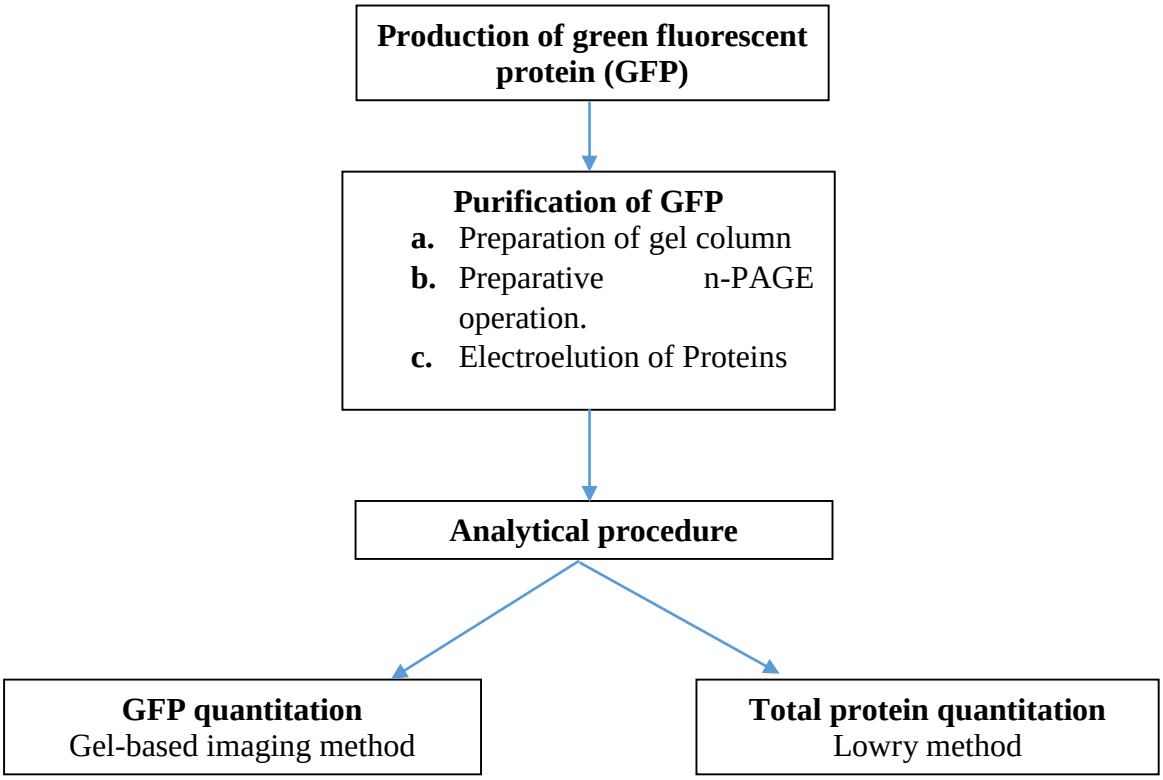

Figure 3.1: Process flow of methodology

3.2 Chemicals

Table 3.1: List of chemicals

Chemical	**Supplier**	**Purpose**
Pure GFP	-	Standard Curve
LB Broth (Lennox)	Condo Pronadisa	Fermentation
LB Agar (Lennox)	Condo Pronadisa	Fermentation
Ampicillin	Bio Basic Canada Inc.	Fermentation
Isopropyl β-D-1-thiogalactopyranoside (IPTG)	Thermo-Scientific	Fermentation
Acrylamide	Merck	Purification
Bis-acrylamide	Bio Basic Canada Inc.	Purification
TRIS	Sigma-Aldrich	Purification
Glycine	Fisher Scientific	Purification
N,N,N',N'-tetramethyl ethylenediamine (TEMED)	Merck	Purification
Ammonium persulfate	Merck	Purification
Bromophenol blue	Fisher Scientific	Purification
Lowry Reagent (Reagent 1)	R&M Chemicals	Quantitation
Folin & Ciocalteu's phenol reagent	Sigma-Aldrich	Quantitation

3.3 Production of green fluorescent protein (GFP)

The experiment began with streaking the *E. coli* strain BL21 (DE3) into the agar plate and incubated for 18 hours in 37 °C. Luria Bertani (LB) broth containing 10 g/L tryptone, 5 g/L yeast extract, 5 g/L sodium chloride, and 100 µg/ml ampicillin was used as the culture medium in this experiment. The ratio of the medium to the Erlenmeyer flask volume was 0.2 in order to provide good oxygen transfer rate during the fermentation process. The inoculum was prepared from a single colony of E. coli from agar plate and transferred into 100 mL of Erlenmeyer flask containing 20 mL medium. The condition for inoculum fermentation process was at 30°C under 200 rpm for 18 hours using a shaker incubator (INFORS HT, *Ecotron*). After 18 hours of fermentation, the process of GFP incubation continued by transferring 1:25 of inoculum into 1000 mL of Erlenmeyer flask containing 200 mL medium. 0.5mM of IPTG was added after about 1 hour and 45 minute (OD_{600}= 0.8-1.0) of fermentation process. The fermentation process continued for another 16 hours at 30°C under 200 rpm.

After 16 hours of cultivation, the cells were harvested by centrifugation at 5800 rpm, 4°C for 30 min using a refrigerated centrifuge (Eppendorf, Centrifuge 5810R). Then, the cell pellets were washed in sample buffer and followed by centrifugation at the same conditions. The cells were suspended in sample buffer as the preparation for the next process.

3.4 Purification of GFP

3.4.1 Preparation of native polyacrylamide gel electrophoresis (n- PAGE) column

Before filling the gel column with polyacrylamide solution, the bottom of the gel column was sealed tightly with parafilm to avoid the leakage of the solution during filling the solution into gel column. 12% (w/v) of resolving gel mixture (Table 3.2) was prepared and loaded into the gel column. 200 µL of saturated butanol was added into the solution in order to form a uniform flat surface. Then, the solution was allowed to polymerise for 30 minutes at room temperature. After the gel was polymerised, the saturated butanol was rinsed thoroughly with distilled water. The homemade gel electrophoresis apparatus was assembled according to the Figure 3.2.

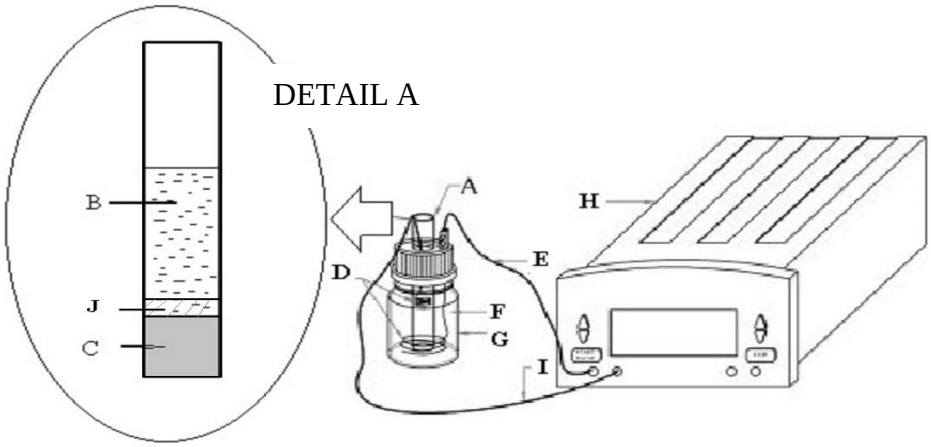

Figure 3.2: Homemade gel electrophoresis apparatus. (A) = Column; (B) = Cathode chamber; (C) = Resolving gel; (D) = platinum wire electrodes; (E) = Anode wire; (F) = Anode chamber; (G) = Laboratory bottle; (H) = Power supply; (I) = Cathode wire; (J) = Loaded sample

Table 3.2: Resolving gel formulation

Components	**Volume (µL)**
Acrylamide mix [30% (w/v) acrylamide and 0.8% (w/v) bisacrylamide]	1200
Distilled water	1050
4x native lower buffer[1.5 M Tris hydrochloride (pH 8.8)]	750
10% (w/v) ammonium persulfate	18.75
N,N,N’,N’-tetramethylethylenediamine	3.03